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Abstract

The carbohydrate-binding specificities of lectins in solution to glycoproteins and neoglycolipids immobilized on a solid phase were analyzed in order to establish a simple, rapid method for structural analysis of the carbohydrate moieties of small amounts of individual glycoproteins blotted on membrane. Eight glycoproteins containing typical O-linked tetrasaccharides or a series of typical N-linked oligosaccharides of the high-man-nose type, hybrid type, and complex type and 6 neoglycoproteins containing mono- or di-saccharides were dot blotted on membranes and the membranes were then reacted with 8 kinds of horseradish peroxidase-conjugated lectins before and after heat treatment. Neoglycolipids containing the glycoprotein-derived oligosaccharides immobilized on a thin layer chromatography plate were also reacted with lectins. The heat treatment of the membrane increased lectin reactivity toward the glycoproteins. The carbohydrate-binding behavior of lectins, Phaseolus vulgaris erythroagglutinin, wheat germ agglutinin, and concanavalin A in solution toward glycoproteins and neoglycolipids immobilized on a solid phase differed from that of immobilized lectins toward oligosaccharides in solution. This difference should be noted in lectin detection of specific carbohydrates of individual glycoproteins on membrane.

KEYWORDS: glycoprotein, lectins, lectin binding specificity, neoglycolipid, oligosaccharide

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The carbohydrate-binding specificities of lectins in solution to glycoproteins and neoglycolipids immobilized on a solid phase were analyzed in order to establish a simple, rapid method for structural analysis of the carbohydrate moieties of small amounts of individual glycoproteins blotted on membrane. Eight glycoproteins containing typical O-linked tetrasaccharides or a series of typical N-linked oligosaccharides of the high-mannose type, hybrid type, and complex type and 6 neoglycoproteins containing mono- or di-saccharides were dot blotted on membranes and the membranes were then reacted with 8 kinds of horseradish peroxidase-conjugated lectins before and after heat treatment. Neoglycolipids containing the glycoprotein-derived oligosaccharides immobilized on a thin layer chromatography plate were also reacted with lectins. The heat treatment of the membrane increased lectin reactivity toward the glycoproteins. The carbohydrate-binding behavior of lectins, *Phaseolus vulgaris* erythroagglutinin, wheat germ agglutinin, and concanavalin A in solution toward glycoproteins and neoglycolipids immobilized on a solid phase differed from that of immobilized lectins toward oligosaccharides in solution. This difference should be noted in lectin detection of specific carbohydrates of individual glycoproteins on membrane.

Key words: glycoprotein, lectins, lectin binding specificity, neoglycolipid, oligosaccharide

The lectin, a family of carbohydrate-binding proteins, is widely distributed in bacteria, plants, and animals. Lectins are thought to play roles in various physiological phenomena, such as cell-cell interaction, via their carbohydrate-binding specificities (1). Lectins, especially plant lectins, have also been used for separation of oligosaccharides and glycoproteins. The carbohydrate-binding specificities of lectins toward oligosaccharides have been demonstrated by affinity chromatography using lectin immobilized on gel and oligosaccharide derived from glycoproteins dissolved in solution (2). Utilizing carbohydrate-binding specificities, unknown structures of oligosaccharides obtained from glycoproteins can be analyzed through various combinations of affinity chromatography using several immobilized lectins (2-4). The lectin blotting method was recently developed as a rapid, convenient method for analysis of the carbohydrate moieties of glycoproteins (5, 6). In this method, glycoproteins are transferred and immobilized on membrane (*e.g.* nitrocellulose or polyvinylidene difluoride (PVDF) membrane), and then reacted with horseradish peroxidase-conjugated lectins in solution. This is a simple, useful method for analysis of the carbohydrate moieties of crude glycoproteins related to various biological phenomena, even in small amounts. However, the information about the carbohydrate-binding specificities of lectins in solution toward glycoproteins and oligosaccharides immobilized on membrane is limited and isolated.

To apply lectins in the rapid analysis of the structures of oligosaccharides in glycoproteins, it is important to clarify the binding preferences of lectins toward glycoproteins transferred onto membrane. In this paper, we examine the carbohydrate-binding specificities of 8 kinds of lectins, *Ricinus communis* agglutinin (RCA120), *Allomyrina dichotoma* agglutinin (Allo-A), *Erythrina cristagalli* agglutinin (ECA), peanut agglutinin (PNA), wheat germ agglutinin (WGA), concanavalin A (Con A), *Lens*

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culinaris agglutinin (LCA), and *Phaseolus vulgaris* erythroagglutinin (PHA-E4), toward various immobilized glycoproteins with an array of known oligosaccharide structures as well as immobilized neoglycolipids prepared from their oligosaccharides. Based on the behavior of oligosaccharides in solution toward the lectin-agarose column, the carbohydrate specificities of the lectins under study were shown to be as follows: RCA120, Allo-A, and ECA recognize Galβ1-4GlcNAc (7-10), but PNA reacts to Galβ1-3GalNAc (11); Con A binds to high-mannose, hybrid, and biantennary complex types of oligosaccharides (12, 13); WGA can recognize terminal GlcNAc (14), or clustered sialic acid (15), and also hybrid type oligosaccharides (16); LCA reacts with bi- and tri-antennary complex-type oligosaccharides with fucose α1,6-linked to proximal GlcNAc (17); and PHA-E4 recognizes bi- and tri-antennary complex-type oligosaccharides with bisecting GlcNAc (18, 19). Our results indicate that the carbohydrate-binding specificities of some of these lectins toward immobilized glycoproteins and immobilized oligosaccharides differ from those shown so far in lectin-agarose column chromatography using oligosaccharide solution.

Materials and Methods

Glycoproteins and lectins. Horseradish peroxidase (HRP)-conjugated RCA120, PNA, PHA-E4, WGA, Con A, and LCA were purchased from Honen Corp., Tokyo. HRP-conjugated Allo-A and ECA, mannose-conjugated bovine serum albumin (Man-BSA), glucose-BSA (Glc-BSA), *N*-acetylglucosamine-BSA (GlcNAc-BSA), and galactose-BSA (Gal-BSA) were obtained from E. Y. laboratories, Inc., San Mateo, CA. Maltose-BSA (Mal-BSA), lactose-BSA (Lac-BSA), bovine pancreatic ribonuclease B, ovalbumin, bovine fetuin and bovine asialofetuin, human IgG, human transferrin, human α_1-acid glycoprotein, and human lactoferrin were purchased from Sigma-Aldrich Japan, Tokyo. Amounts of carbohydrates in Man-BSA, Glc-BSA, GlcNAc-BSA, Gal-BSA, Mal-BSA, and Lac-BSA were 23, 42, 30, 34, 14, and 19 moles/1 mole of BSA, respectively. Human fibrinogen and human IgM were obtained from AB KABI, Stockholm and Green Cross Corp., Osaka, respectively. Galactose-lacking IgG (Gal-lacking IgG) was prepared from human IgG by digestion with sialidase and β-galactosidase (20). *Arthrobacter ureafacience* sialidase was purchased from Nacalai Tesque Inc., Kyoto.

Dot blotting. The PVDF membrane (0.2 μm) was obtained from Bio-Rad Laboratories, Hercules, CA. The membrane was dipped in methanol, equilibrated with water, and then clamped in a dot-blotter. Six neoglycoproteins (Man-BSA, Glc-BSA, GlcNAc-BSA, Gal-BSA, Mal-BSA, and Lac-BSA) and eight natural glycoproteins with known oligosaccharide structures (human IgM (21), human IgG (22), Gal-lacking IgG (20), human fibrinogen (23), bovine fetuin (24), bovine asialofetuin (24), human lactoferrin (25), ovalbumin (26)), were dissolved in PBS at a concentration of 100 μg/ml. Fifty μl of each solution was added to wells. After 15 min, the solution in the well was removed by aspiration and the wells were washed 5 times with Tris-buffered saline (TBS). Triplicate membranes were prepared for reaction with each lectin. One was stained with Coomassie Brilliant Blue to confirm adsorption of the glycoproteins on the membrane. The rest were used for lectin reaction before and after heat treatment at 100°C for 10 min.

Detection of glycoproteins. The membrane was blocked with TBS containing 3% BSA, and then reacted with HRP-conjugated lectin dissolved in TBS containing 1% BSA for 1 hr at room temperature. After being washed 4 times with TBS containing 0.1% Tween 20, the membrane was visualized with 0.05% 4-chloro-1-naphthol and 0.01% H_2O_2. The reaction was terminated by washing the membrane with water. PHA-E4, Con A, LCA, WGA, and PNA were used at a concentration of 2 μg/ml. RCA120, Allo-A, and ECA were used at concentrations of 0.5, 3, and 5 μg/ml, respectively.

Conversion of oligosaccharides into neoglycolipids and detection of neoglycolipids on thin layer chromatography plates. The following six glycoproteins with known oligosaccharide structures were used as sources of *N*-linked oligosaccharides: ovalbumin (26), bovine pancreatic ribonuclease B (27), human transferrin (28), human IgG (22), bovine fetuin (24), and human α_1-acid glycoprotein (29). The quantitative release of *N*-linked oligosaccharides from these glycoproteins by hydrazinolysis was performed as described previously in detail (30, 31). Part of the oligosaccharide mixtures obtained from transferrin, IgG, fetuin, and α_1-acid glycoprotein was converted into neutral oligosaccharides by sialidase treatment as described previously (30). Oligosaccharide mixtures obtained from glycoproteins and the desialylated neutral oligosaccharide mixtures were conjugated to dipalmitoylphosphatidylethanolamine to prepare neoglycolipids as described previous-

ly (32, 33). The reaction mixtures containing 2.5 μg of oligosaccharides were subjected to thin layer chromatography (TLC) using high-performance aluminium-backed silica gel TLC plates, Art 5,547 from Merck, Darmstadt and development solvent of chloroform/methanol/water (105:100:28, by volume). Separation of neoglycolipids on the TLC plate was confirmed by spraying 0.001 % primulin solution dissolved in acetone-water (4:1, by volume) followed by viewing under long-wavelength ultraviolet light. The TLC plates on which the neoglycolipids were separated based on their oligosaccharide structures were then overlaid with HRP-conjugated lectins (2 μg/ml) and incubated for 1 hr at room temperature. After the plate was washed, binding of lectins to neoglycolipids was visualized by 4-chloro-1-naphthol. The conditions for this lectin overlay assay were basically the same as those for the detection of glycoproteins on a PVDF membrane described above. Other conditions were according to those described previously (32, 34, 35).

Results and Discussion

The binding specificities of lectins to immobilized synthetic neoglycoproteins (Man-BSA, Glc-BSA, GlcNAc-BSA, Gal-BSA, Mal-BSA, and Lac-BSA) and natural glycoproteins (ovalbumin, IgM, IgG, Gal-lacking IgG, fibrinogen, fetuin, asialofetuin, and lactoferrin) were tested. The structures of a series of typical *N*-linked oligosaccharides occurring in natural glycoproteins are summarized in Fig. 1. IgM contains high-mannose type oligosacharides and biantennary complex-type oligosaccharides with and without a bisecting GlcNAc (21). Ovalbumin contains high-mannose type and hybrid type oligosaccharides (26). IgG contains di-, mono-, and non-galactosylated biantennary complex-type oligosaccharides with and without a bisecting GlcNAc (22). Fibrinogen contains sialylated biantennary complex-type oligosaccharides without fucose (23). Lactoferrin contains sialylated biantennary complex-type oligosaccharides with and without the Le^x structure of Galβ1-4(Fucα1-3)GlcNAc as the major oligosaccharide (25).

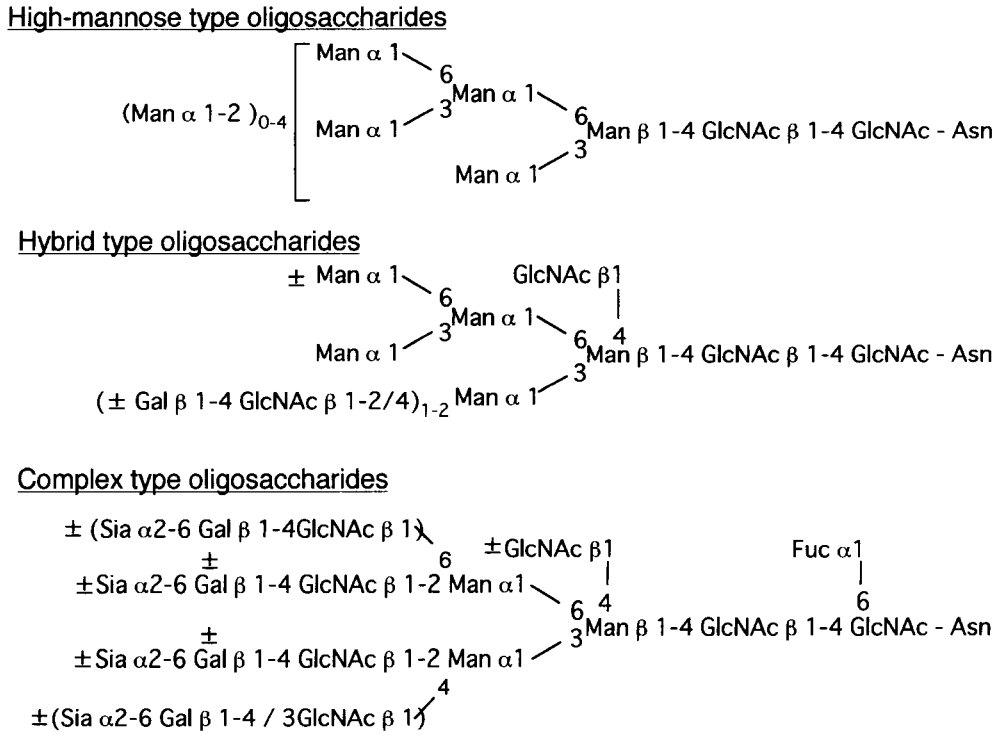

Fig. 1 Structures of *N*-linked oligosaccharides carried on glycoproteins.

Fetuin contains sialylated bi- and tri-antennary complex-type oligosaccharides without fucose in addition to typical *O*-linked oligosaccharides, Sialic acidα2-3Galβ1-3(Sialic acidα2-6)GalNAc (24). Asialofetuin contains asialo-structures of fetuin oligosaccharides. Gal-lacking IgG contains asialo-, agalacto-, and GlcNAc-exposing structures of the IgG oligosaccharides (20).

The glycoproteins were transferred onto a PVDF membrane by dot blotting. The membrane was then reacted with 8 kinds of HRP-conjugated lectins whose carbohydrate-binding specificities had been demonstrated by lectin-agarose column chromatography for oligosaccharides. To estimate the effect of conformation of glycoproteins immobilized on membrane on lectin reactivity, we compared the binding of lectins to immobilized glycoproteins before and after treatment of the membrane at 100°C for 10 min. As shown in Fig. 2, ECA did not bind to IgG before the membrane was heat treated, but recognized it after the treatment. An increase in the reactivity of lectin to IgG due to heat treatment was also observed in the case of RCA120, Allo-A, PHA-E4, Con A, and LCA. Heat denaturation of IgM and ovalbumin also increased the binding ability of RCA120, Con A, and WGA. No degradation of oligosaccharides derived from the glycoproteins by heat treatment was observed by their HPLC analyses using DEAE- and ODS-columns (data

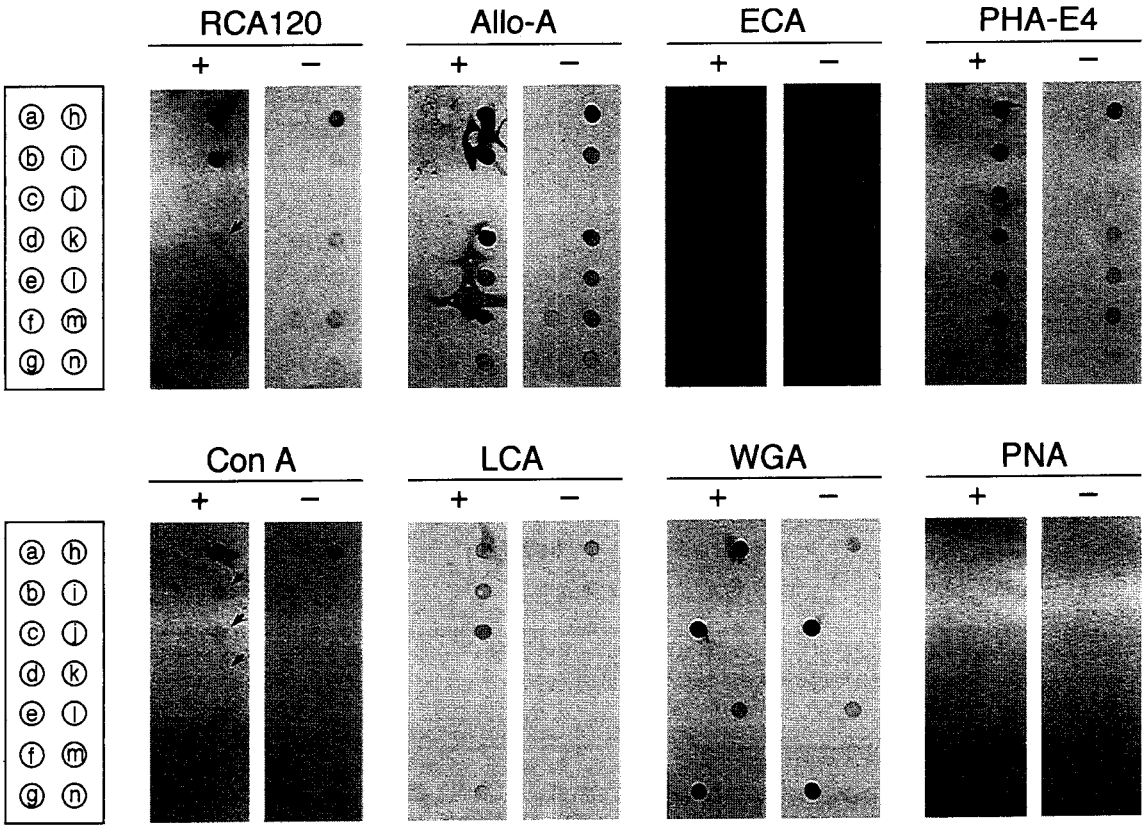

Fig. 2 Detection by lectin in the solution of glycoproteins immobilized on a membrane. The glycoproteins were dot-blotted onto a polyvinylidene difluoride (PVDF) membrane and then reacted with horseradish peroxidase (HRP)-lectins before (−) and after (+) heat treatment at 100°C for 10 min, as described in the Materials and Methods section. The following glycoproteins were dot-blotted on each membrane; a: Mannose-conjugated bovine serum albumin (BSA); b: Glucose-BSA; c: *N*-acetylglucosamine-BSA; d: Galactose (Gal)-BSA; e: Maltose-BSA; f: Lactose-BSA; g: Ovalbumin; h: Human IgM; i: Human IgG; j: Human Gal-lacking IgG; k: Human fibrinogen; l: Bovine fetuin; m: Bovine asialofetuin; n: Human lactoferrin. Arrows indicate dots faintly reactive with HRP-lectins. RCA120: *Ricinus communis* agglutinin; Allo-A: *Allomyrina dichotoma* agglutinin; ECA: *Erythrina cristagalli* agglutinin; PHA-E4: *Phaseolus vulgaris* erythroagglutinin; Con A: Concanavalin A; LCA: *Lens culinaris* agglutinin; WGA: Wheat germ agglutinin; PNA: Peanut agglutinin.

not shown). Therefore, heat treatment of glycoproteins immobilized on membrane was useful in increasing the sensitivity of detection by lectins of the specific carbohydrates of glycoproteins.

RCA120, Allo-A, and ECA, classified into β-galactoside-binding lectins, recognized IgM, IgG, fibrinogen, asialofetuin, and lactoferrin (Fig. 2), which contained Galβ1-4GlcNAc structures in their oligosaccharides (Fig. 1). As can be seen in Fig. 2, Allo-A exhibited much broader binding-specificity than RCA120 and ECA. Since fetuin was recognized by Allo-A but not by RCA120 and ECA, and since asialofetuin was recognized by all three, it appears that Allo-A could recognize not only terminal galactose residue but also internal galactose residue linked with sialic acid when glycoproteins are immobilized on a solid phase. The binding of LCA to IgM, IgG, Gal-lacking IgG, and lactoferrin and the lack of binding to the other immobilized glycoproteins tested (Fig. 2) were consistent with the carbohydrate-binding specificities which have been shown to recognize bi- and tri-antennary oligosaccharides with fucose α1,6-linked to proximal GlcNAc in LCA-agarose column chromatography (17). As expected, PNA reacted only to asialofetuin with *O*-glycosidically linked Galβ1-3GalNAc, which is strongly recognized by PNA-agarose (Fig. 2).

In contrast, the binding behavior of PHA-E4, Con A, and WGA in solution toward immobilized glycoproteins differed from the binding specificities thus far reported for lectins immobilized on agarose gel toward oligosaccharides in solution. PHA-E4 clearly reacted with all the natural glycoproteins tested, including fetuin, asialofetuin, fibrinogen, and lactoferrin, which do not carry bisecting GlcNAc-containing oligosaccharides (Fig. 2). This was the case even though it is well known that PHA-E4 immobilized on agarose gel has affinity only for oligosaccharides with bisecting GlcNAc (18, 19) and does not recognize oligosaccharides prepared from asialofetuin (19). Con A strongly recognized only IgM and ovalbumin, which contained high-mannose type or hybrid type oligosaccharides, but faintly bound to IgG, Gal-lacking IgG, fibrinogen, and lactoferrin only after the heat treatment (Fig. 2) even though they contain biantennary complex-type oligosaccharides. This behavior of Con A toward immobilized glycoproteins with biantennary oligosaccharides on membrane differed from that of Con A-agarose toward oligosaccharides in solution, since Con A-agarose clearly binds not only high-mannose type and hybrid type oligosaccharides but also biantennary complex-type oligosaccharides derived from those glycoproteins, and releases them in the presence of α-methyl-D-mannoside (12, 13). WGA-agarose can recognize hybrid type oligosaccharides (16), clustered sialic acid (15), and terminal GlcNAc (14). WGA consistently bound to ovalbumin, fetuin, and GlcNAc-BSA immobilized on the membrane (Fig. 2), which contain hybrid-type oligosaccharides, sialylated triantennary complex-type oligosaccharides, and multiple terminal GlcNAc residues, respectively. However, WGA unexpectedly could not bind to Gal-lacking IgG (Fig. 2), although it exposed GlcNAc residues at the non-reducing termini of the oligosaccharides. These discrepancies may be ascribed to a difference in lectin binding affinities between those for oligosaccharides and those for glycoproteins with the same oligosaccharides, or to a difference in lectin affinities for oligosaccharides in different states *i.e.*, oligosaccharides and glycoproteins immobilized on solid phase or dissolved in aqueous solution.

To elucidate whether or not the affinity of lectins for glycoproteins is the same as that for oligosaccharides, the reactivity of lectins to oligosaccharide derivatives (neoglycolipids) immobilized on a TLC plate was examined and compared with reactivity to glycoproteins immobilized on a PVDF membrane. The neoglycolipids used here were prepared using oligosaccharides (Fig. 1) derived from ribonuclease with a series of high-mannose type oligosaccharides (27), transferrin with sialylated biantennary complex-type oligosaccharides without fucose as the major oligosaccharides (28), α_1-acid glycoprotein with sialylated bi-, tri-, and tetra-antennary complex-type oligosaccharides without fucose (29), ovalbumin, IgG, and fetuin. As shown in Fig. 3, biantennary complex-type neoglycolipids prepared from asialo oligosaccharides of IgG and transferrin faintly reacted with Con A, as in the case of immobilized IgG (Fig. 2), while hybrid-type and high-mannose type neoglycolipids derived from ovalbumin and ribonuclease clearly reacted, as in the case of immobilized ovalbumin (Fig. 2). WGA strongly recognized hybrid-type neoglycolipids prepared from oligosaccharides of ovalbumin and weakly recognized sialylated neoglycolipids from fetuin and α_1-acid glycoprotein, while it did not bind to neoglycolipids with sialylated biantennary oligosaccharides of transferrin (Fig. 3), as in the case of immobilized glycoproteins (Fig. 2). Neoglycolipids with biantennary complex-type oligosaccharides exposing non-reducing terminal GlcNAc residues from IgG could not be detected by WGA (Fig. 3), as in the case of immobilized Gal-lacking (GlcNAc-exposing) IgG (Fig. 2). PHA-E4

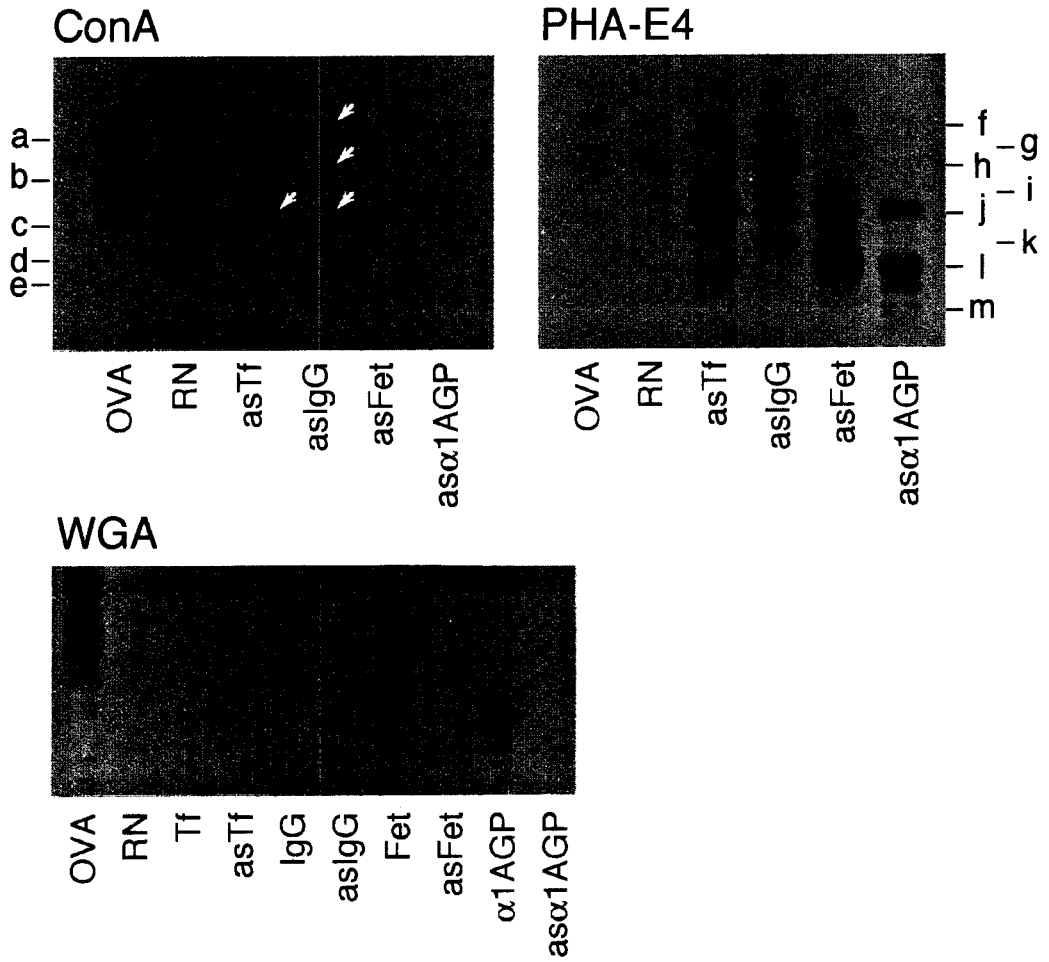

Fig. 3 Reactivity of immobilized oligosaccharides released from glycoproteins after conversion of oligosaccharides into neoglycolipids. Neoglycolipids prepared from *N*-linked oligosaccharides released from glycoproteins were separated by thin layer chromatography (TLC). The TLC plates were overlaid by HRP-lectins as described in the Materials and Methods section. OVA, RN, Tf, IgG, Fet, and α_1AGP indicate lanes where neoglycolipids prepared from oligosaccharides released from ovalbumin, ribonuclease, transferrin, IgG, fetuin, and α_1-acid glycoprotein, respectively, were separated. asTf, asIgG, asFet, and asα_1AGP indicate lanes where neoglycolipids prepared from desialylated oligosaccharides derived from transferrin, IgG, fetuin, and α_1-acid glycoprotein, respectively, were separated. White arrows indicate bands faintly reactive with HRP-lectins. Bars a-m in the figure indicate the positions where neoglycolipids prepared from the following authentic oligosaccharides moved: a-e: A series of high mannose type oligosaccharides, $Man_{5-9}GlcNAc_2$; f: GlcNAcβ1-2Manα1-6(GlcNAcβ1-2Manα1-3)Manβ1-4GlcNAcβ1-4(Fucα1-6)GlcNAc; g: GlcNAcβ1-2Manα1-6(GlcNAcβ1-4)(GlcNAcβ1-2Manα1-3)Manβ1-4GlcNAcβ1-4(Fucα1-6)GlcNAc; h: Galβ1-4GlcNAcβ1-2Manα1-6/3(GlcNAcβ1-2Manα1-3/6)Manβ1-4GlcNAcβ1-4(Fucα1-6)GlcNAc; i: Galβ1-4GlcNAcβ1-2Manα1-6/3(GlcNAcβ1-4)(GlcNAcβ1-2Manα1-3/6)Manβ1-4GlcNAcβ1-4(Fucα1-6)GlcNAc; j: Galβ1-4GlcNAcβ1-2Manα1-6(Galβ1-4GlcNAcβ1-2Manα1-3)Manβ1-4GlcNAcβ1-4(Fucα1-6)GlcNAc; k: Galβ1-4GlcNAcβ1-2Manα1-6(GlcNAcβ1-4)(Galβ1-4GlcNAcβ1-2Manα1-3)Manβ1-4GlcNAcβ1-4(Fucα1-6)GlcNAc; l: Galβ1-4GlcNAcβ1-2Manα1-6[Galβ1-4GlcNAcβ1-4(Galβ1-4GlcNAcβ1-2)Manα1-3]Manβ1-4GlcNAcβ1-4GlcNAc; m: Galβ1-4GlcNAcβ1-6(Galβ1-4GlcNAcβ1-2)Manα1-6[Galβ1-4GlcNAcβ1-4(Galβ1-4GlcNAcβ1-2)Manα1-3]Manβ1-4GlcNAcβ1-4GlcNAc. Con A; PHA-E4; WGA: See legend to Fig. 2.

strongly bound to bi-, tri-, and tetra-antennary complex-type neoglycolipids without bisecting GlcNAc prepared from transferrin, fetuin, and α_1-acid glycoprotein on a TLC plate as well as to biantennary complex-type neoglycolipids with bisecting GlcNAc from IgG (Fig. 3). These results strongly suggest that the reactivity of lectins dissolved in solution to oligosaccharides immobilized on a solid phase was similar to that to immobilized

glycoproteins with the corresponding oligosaccharides, but that it differed from the reactivity of lectins immobilized on a solid phase to oligosaccharides dissolved in solution.

Recently, understanding of the involvement of specific carbohydrate structures on glycoproteins with cell-cell interactions and diseases has been increasing. However, the amounts of glycoproteins involved in these phenomena are usually very low, and therefore, determination of their carbohydrate structures is difficult. To overcome this problem, it would be useful to detect the carbohydrate moieties of individual glycoproteins using a variety of lectins with affinities for specific carbohydrates, after separation of crude glycoproteins by SDS-PAGE followed by transfer to membrane. To establish such a method, information on lectin reactivity to oligosaccharides and glycoproteins immobilized on a solid phase is essential. This study indicates that the carbohydrate-binding behavior of certain lectins toward glycoproteins and their oligosaccharides immobilized on solid phase differs from the behavior of the immobilized corresponding lectins toward oligosaccharides in solution thus far demonstrated by lectin-agarose column chromatography.

This difference should be noted upon detection by lectins of specific carbohydrates in individual glycoproteins on membrane. Indeed, some lectins have been already used for detection of specific structures of oligosaccharides of glycoproteins on membrane, based on the binding specificities of lectin-agarose toward oligosaccharides in solution (36-38). Based on the results obtained in this study, Con A especially should be used for limited detection of high-mannose and hybrid type oligosaccharide-carrying glycoproteins on membrane, and WGA for hybrid-type or sialic acid-clustering oligosaccharide-carrying glycoproteins on membrane. LCA can be used for definite detection of α1,6-linked fucose-containing oligosaccharide-carrying glycoproteins. However, the use of PHA-E4 for detection of glycoproteins with bisecting GlcNAc-containing oligosaccharides on membrane should be strictly controlled. When other lectins not tested in this paper are used to obtain structural information on the carbohydrate moieties of glycoproteins immobilized on a solid phase, it will be necessary to examine their carbohydrate-binding preferences toward immobilized glycoproteins or neoglycolipids. Detection of specific carbohydrates on glycoproteins by lectins after conversion of oligosaccharides into neoglycolipids may be another powerful method for analysis of the oligosaccharide moieties of glycoproteins.

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